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Polyphenols of marine red macroalga *Symphyocladia latiuscula* ameliorate diabetic peripheral neuropathy in experimental animals

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ABSTRACT

Aims: Chronic hyperglycaemia activates the polyol pathway of glucose metabolism thereby stimulating the activation aldose reductase enzyme that in turn initiates a cascade of deleterious events, eventually, leading to nerve damage or neuropathy. Marine macroalgae and their isolated chemical constituents have been found to possess potential antidiabetic activity and have proved beneficial in the treatment of diabetes. In this study the neuroprotective effect of polyphenols isolated from the red macroalga *Symphyocladia latiuscula* was evaluated in experimental diabetic peripheral neuropathy.

Main methods: The polyphenolic fraction from *Symphyocladia latiuscula* was isolated. Diabetic peripheral neuropathy (DPN) was induced in animals by intraperitoneal injection of streptozotocin (45 mg/kg, b. w) and maintained for 6 weeks followed by treatment with SLPP or epalrestat. Nerve Conduction Velocity (NCV) and Compound Muscle Action Potential (CMAP) were measured using a non-invasive method followed by muscular grip strength test. Sciatic nerve aldose reductase activity, sorbitol accumulation, Na^+K^+-ATPase activity, production of pro-inflammatory cytokines and expression of AR and PKC were assessed.

Key findings: The *Symphyocladia latiuscula* polyphenols (SLPP) were found to inhibit aldose reductase activity as well as their expression in diabetic animals thereby improving the NCV, CMAP and muscle grip strength. Improvements in the sciatic nerve Na^+K^+-ATPase activity and intraneural accumulation of sorbitol, an index of aldose reductase overactivity, were evident with SLPP treatment. The production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) and expression of protein kinase C (PKC) were also diminished.

Significance: The data suggest that the polyphenols of *Symphyocladia latiuscula* have neuroprotective potential against experimental DPN.

1. Introduction

Neuropathy is the commonest microvascular complication affecting around 30 million people throughout the world and is a prominent source of mortality and morbidity. Hyperglycaemia is one of the primary issues leading to neuropathy [1]. Neurons have a persistently elevated demand of glucose and uptake depends primarily on its extracellular concentration. They can neither afford anaerobic and glycolytic events nor can they put up with intermittent insulin influenced glucose uptake. Hyperglycaemia in diabetes causes the neuronal glucose to rise up to four folds. Such events, if persistent or frequent, may lead to neuronal damage owing to intracellular metabolism of glucose; this incidence is commonly called glucose neurotoxicity [2].

The polyol pathway of glucose metabolism plays crucial function in developing neuropathy [3]. Polyol pathway over-activity [4, 5] and enhanced non-enzymatic glycation [6, 7] have been implicated in diabetic neuropathy. It is an alternate route of glucose metabolism in which the enzyme aldose reductase catalyzes the reduction of glucose to sorbitol, then to fructose by sorbitol dehydrogenase. Aldose reductase (AR) requires NADPH as co-factor and sorbitol dehydrogenase (SDH) needs NAD^+. During hyperglycemia, sorbitol accumulates in AR-containing tissues as it is impermeable to the cell membranes and cannot diffuse out and, hence, creates hyperosmotic stress on the cell thereby inducing neuropathic pain [8, 9]. Treatment with inhibitors of aldose reductase has shown prevention of various complications including nephropathy, neuropathy, and cataract in animal models [10]. Accumulation of intracellular sorbitol and fructose leads to diminution of other organic electrolytes like taurine and *myo*-inositol that regulate cellular osmolality [11]. Lessening of *myo*-inositol in the peripheral nerves interferes with the production of phosphoinositide producing inadequate diacylglycerol

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to sustain the content of protein kinase C (PKC) essential for Na^+K^+-ATPase activation [12, 13]. Amendments in PKC activation also interfere with an important myelin protein's (PO) phosphorylation of peripheral nerve and deliberate an important pathogenetic role in primary segmental demyelination. Enhanced activity of vascular PKC-β is thought to play a noteworthy responsibility in microvascular complications [14]. Oxidative stress has also been critically implicated in the development of neuropathy [15]. These abnormalities initiate a chronic progressive damage and loss in unmyelinated and myelinated peripheral nerve fibers that culminate in peripheral polyneuropathy [16, 17].

Polyphenols isolated from various marine macroalgae have been found to possess anti-diabetic activities [18, 19, 20]. The red alga *Symphyocladia latiuscula* (Harvey) Yamada of the family Rhodomelaceae is known to contain high concentrations of bromophenols that were reported to possess free-radical scavenging [21, 22], antibacterial [23], antiviral [24], anticancer [25] and α-glucosidase inhibition activities [26]. The polyphenolic constituents of *Symphyocladia latiuscula* have been isolated, characterized and reported by various researchers [27, 28], and hence, we have not attempted the same. Instead, its neuroprotective activity in diabetic peripheral neuropathy that was not elaborated elsewhere was explored. In this study, we investigated the effects of SLPP on nerve conduction velocity (NCV), compound muscle action potential (CMAP), aldose reductase activity and intraneural sorbitol accumulation in peripheral nerves (sciatic nerve). The expressions of AR, PKC and pro-inflammatory cytokines were also studied.

2. Materials and methods

2.1. Chemicals and reagents

Streptozotocin, RIPA buffer and protease inhibitor cocktail tablets (SigmaFAST™) were procured from Sigma-Aldrich (USA). DL-glyceraldehyde and NADPH was obtained from Himedia, Mumbai, India. Primary antibodies for IL-6, IL-1β, TNF-α and rabbit anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, USA. Primary antibodies for aldose reductase, PKC and Goat Anti-Rabbit IgG-HRP were procured from Abcam, USA. Cell culture media and reagents were obtained from Gibco, Thermo Scientific. Solvents and chemicals were of EMPLURA® and extra pure grades from Merck. Epalrestat, an aldose reductase inhibitor, was received as a generous gift from Zydus Cadila, India.

2.2. Experimental animals

Wistar rats weighing between 160-220 g were maintained in standard laboratory conditions at room temperature (25 ± 2 °C) with a 12-hour light/12-hour dark cycle. The animals were given pellet chow and water *ad libitum* except during experimentation. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) bearing registration number 1564/PO/Re/S/11/CPCSEA, and performed in accordance with the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals (NIH Publication No. 8023).

2.3. Isolation of *Symphyocladia latiuscula* polyphenols

The *Symphyocladia latiuscula* (SL) were procured from South China coast through a reputed commercial dealer and authenticated. Dried, fine powders of SL were subjected to continuous hot extraction with 70% methanol for 3 h with reflux at 70–75 °C three times successively. The extract was concentrated to half its volume and partitioned with n-hexane (five times) to remove pigments & lipids. Aqueous fraction contained soluble polyphenols (positive with Folin-Ciocaulteu's phenol reagent) that were precipitated with acetonitrile (1:1), concentrated in a rotary evaporator and lyophilized to obtain light brown crystals. The polyphenol fraction was designated *Symphyocladia latiuscula* polyphenols (SLPP).

2.4. Determination of polyphenolic concentration

The concentration of polyphenol was determined using the Folin-Ciocaulteu's method [29]. An aliquot (20 μL) of the polyphenol sample (2 mg/mL) was mixed with 250 μL Milli Q water and 250 μL Folin-Ciocaulteu's phenol reagent (Himedia, Mumbai, India). Then, 500 μL of 10% Na_2CO_3 solution was added to the mixture and incubated at room temperature in the dark for 1 h. A series of standard tannic dilutions (10, 20, 40, 60, 80 and 100 μg/mL) were also treated likewise to construct the calibration curve. The absorbance against a blank was measured at 750 nm. Polyphenol concentration was calculated from the standard calibration curve.

2.5. Induction of peripheral neuropathy

Wistar rats were rendered diabetic with streptozotocin (45 mg/kg) injection (i.p.) and maintained for 6 weeks. The animals were grouped (n = 6) as: **Group I**: Normal control (untreated); **Group II**: Diabetic Peripheral Neuropathy (DPN) control – STZ (45 mg/kg b. w; i. p); **Group III**: DPN control + SLPP (100 mg/kg; oral); **Group IV**: DPN control + SLPP (200 mg/kg; oral); **Group V**: DPN control + Epalrestat (AR inhibitor; 100 mg/kg) [30]. Induction of neuropathy was determined by measuring the conduction velocity of sciatic nerve by a non-invasive method. Henceforth, the treatment groups were treated with SLPP or epalrestat for 30 consecutive days.

2.6. Electrophysiological measurements (NCV and CMAP)

Nerve conduction velocity (NCV) is used to assess the function, especially the electrical conductance of the sensory and motor nerves. In anesthetized rats motor NCV was recorded from the sciatic nerve of the left tibia through a non-invasive modified method [31, 32]. The nerve was stimulated at the sciatic notch proximally and at the knee distally by bipolar electrodes by AD Instruments (Powerlab data acquisition system, New Zealand). Using unipolar pin electrodes, the compound muscle action potential (CMAP) of the gastrocnemius muscle was recorded from the ankle. The ratio of distance (in millimetre) between both sites of stimulation divided by the difference in time between distal and proximal response time (in milliseconds) gives the NCV (m/s).

2.7. Muscular grip strength test

Muscle relaxation is designated by a loss in muscle grip which occurs in full blown peripheral neuropathy. This effect was studied in animals using a rotating rod (Rotarod, INCO Instruments, India). The difference in time to fall off from the rotating rod between the control and treated animal is taken as an indicator of muscle weakness. The rate of rotation of the rod (20 rpm) was tuned in a way that a normal animal can endure on it for a substantial period (3–5min). The animals were taken through a pre-test on the apparatus. Only those animals that endured for 5 min were selected for the test [33].

2.8. Aldose reductase activity

Animals representing each group (mentioned previously) were euthanized with high dose barbiturate (thiopentone sodium 75 mg/kg; i. p; recommended by the CPCSEA; Annexure 5 & 6). The left sciatic nerve was exposed through a dorsal incision of the thigh and the nerve of full length was removed and transferred to a petri dish containing DMEM supplemented with 10% FBS, Penicillin G (100 IU/mL), Streptomycin sulfate (100 μg/mL) and Amphotericin B (2.5 μg/mL). After rinsing thoroughly the nerves were homogenized with a Polytron homogenizer using a lysis buffer at 0–4 °C containing 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 1% NP40 and a protease inhibitor cocktail (SigmaFAST™, Sigma, USA) containing 2 mM AEBSF, 1 μM Phosphoramidon, 130 μM Bestatin, 14 μM E-64, 1 μM Leupeptin, 0.2 μM Aprotinin and 10 μM Pepstatin A. The lysate was centrifuged and the supernatant was stored at -80 °C till further use. For the determination of the sciatic nerve AR activity, 0.7 mL of phosphate buffer (0.067 M), 0.1 mL of NADPH (25×10^{-5} M), 0.1 mL of homogenate supernatant, 0.1 mL of DL-glyceraldehyde (substrate) (5×10^{-4} M) were taken in a cuvette. Absorbance of the final solution was taken against a reference cuvette containing all components except the substrate, DL-glyceraldehyde. The enzymatic reaction was started by the addition of the substrate and the absorbance (OD) was recorded at 340 nm for 3 min at 30 s interval. The AR activity was expressed as μmoles/min/mL and calculated as per the following equation [34, 35].

$$\text{Units / ml enzyme} = \frac{(\Delta A_{340nm}/\text{minTest} - \Delta A_{340nm}/\text{minBlank}) \times \text{Total assay volume} \times \text{DF}}{\text{Millimolar extinction coefficient of NADPH at 340nm} \times \text{supernatant volume}}$$

where,

DF = Dilution factor; Total volume (in ml) of assay = 3; Volume (in ml) of homogenate supernatant = 0.1; Millimolar extinction coefficient of β-NADPH at 340nm = 6.22.

2.9. Intraneural sorbitol accumulation

The estimation of sorbitol in the TCA-precipitated de-proteinized supernatant was done using an Agilant 1120 HPLC system with a EZChrome software. Separation was done on a Waters Sunfire® C18 reversed-phase column (250 mm × 4.6 mm, 5μm, Milford, MA) and peak detection was performed at 231 nm. A gradient elution was performed with H_2O and acetonitrile (ACN) with flow rate 1.0 mL/min. Volume of analyte injection was 25 μL. Sorbitol reference standard (Sigma-Aldrich, India) was used at a concentration of 100 μg/mL. The gradient program followed was: 0–2 min - H_2O:ACN:30:70; 2–6 min - H_2O:ACN:12.5:87.5; 6–8 min - H_2O:ACN:05:95; 8–9 min - H_2O:ACN:12.5:87.5; 9–10 min - H_2O:ACN:20:80; 10–11 min - H_2O:ACN:30:70 [36, 37]. Sorbitol concentration was determined by quantifying the AUC of sorbitol.

2.10. Measurement of Na^+K^+-ATPase activity

The Na^+ K^+-ATPase activity was assayed in the sciatic nerve lysate by the spectrophotometric determination of inorganic phosphate (Pi) released from ATP, in the presence and absence of ouabain, a specific Na^+ K^+-ATPase antagonist. The lysate was incubated at 37 °C in a reaction mixture containing Tris-HCl (30 mmol/L pH 7.4), EDTA (0.1 mmol/L), NaCl (50 mmol/L), KCl (5 mmol/L), $MgCl_2$ (6 mmol/L), and ATP (1 mmol/L) in the presence or absence of 0.5 mM ouabain [38]. After pre-incubating the homogenate for 10 min at 37 °C, the reaction was started by the addition of ATP and stopped with 50 μL of TCA (30 %) after 20 min. To determine inorganic phosphate (Pi) in the supernatant, 750 μL of a reducing solution containing 3.5 % ferrous ammonium sulphate, 1.0 % thiourea, and 1.0 % H_2SO_4 and 150 μL of an ammonium molybdate solution containing 4.4 % ammonium molybdate and 9 % of H_2SO_4 were added to 750 μL of the solution to be assayed. After 10 min incubation at room temperature, the absorbance at 750 nm was measured with a spectrophotometer and Na^+K^+-ATPase activity was calculated as the difference between the presence or absence of ouabain-sensitive Na^+K^+-ATPase activity [39]. Total protein in the sciatic nerve lysate supernatant was estimated by bicinchoninic (BCA) protein assay kit (BCA-1, Sigma, USA).

2.11. Cytokine ELISA

The production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) was assessed in the sciatic nerve lysate supernatant samples by indirect sandwich ELISA. Wells of ELISA plate (Maxisorp®, NUNC, Denmark) were coated with 100 μL of IL-6 (sc-57315; Santa Cruz Biotech, USA), IL-1β (sc-32294, SCBT) and TNF-α (sc-133192, SCBT) primary (capturing) monoclonal antibodies (2.5 μg mL^{-1}) in carbonate buffer (Na_2HPO_4 and NaH_2PO_4, pH 9.6) and incubated for 12–14 h at 4 °C. The wells were washed (x5) with wash buffer (NaCl and Tween 20 in phosphate buffer, pH 7.4) and blocked with 250 μL of blocking buffer (2% BSA in phosphate buffer, pH 7.4) per well followed by incubation for 1 h at 37 °C. After incubation, standard IL-6 (sc-4597, SCBT), IL-1β (sc-4592, SCBT) and TNF-α (sc-4564, SCBT) were added for the construction of calibration curve. The concentration range used was – 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.39 and 0.195 ng/mL. Remaining wells were coated with 100 μL of appropriately diluted supernatant, incubated for 2 h at 37 °C and washed (x5). After incubating 2 h at 37 °C with the primary (detecting) antibodies (monoclonal mouse anti-IL-6, anti-IL- 1β and anti-TNF-α, 1:1000 in blocking buffer), the wells were washed (x5) and incubated for 1 h at 37 °C with 100 μL of anti-mouse IgG-HRP (monoclonal, 1:5000, sc-358914). Finally, 100 μL of freshly prepared substrate (TMB in DMSO containing H_2O_2) was added to all wells, and incubated in dark 37 °C for 15 min for colour development. The reaction was terminated by adding 50 μL of 2.5 N H_2SO_4 per well and the A_{450nm} was measured using ELISA reader (Robonik, India).

2.12. Western blot analysis

Initially, total protein in the sciatic nerve lysate supernatant was estimated by BCA protein assay kit (BCA-1, Sigma, USA). Aliquots of the lysates containing 40 μg of protein were subjected to denatured SDS-PAGE on polyacrylamide gels (MiniProtean TGX precast gels, BioRad). After transferring onto nitrocellulose membrane, the protein bands were blocked with 10 ml blocking buffer containing 5% non-fat dried milk in TBST (25 mM Tris-HCl, 137 mM NaCl, 2.65 mM KCl, and 0.05% Tween 20; pH 7.4) for 2 h at room temperature. The blots were washed in TBST (x5) and probed with aldose reductase (abcam; ab175394) and protein kinase C (abcam; ab19031) primary antibodies (1:500 dilution block buffer). Beta actin monoclonal antibody (RM112) was used for blot normalization (loading control). After incubating overnight at 4 °C with shaking, anti-rabbit HRP-conjugated secondary antibody (1:2000 dilution) was added and incubated with rocking for 1 h at room temperature. The antibody-reactive bands were visualized by an enhanced chemiluminescence (ECL) detection kit (Pierce, Thermo Scientific).

2.13. Statistical analysis

The results are expressed as mean ± standard error of mean (SEM) and one-way analysis of variance (ANOVA) followed by Dunnett's test was used to determine statistical significance. Values of $p < 0.001$ were considered as statistically significant.

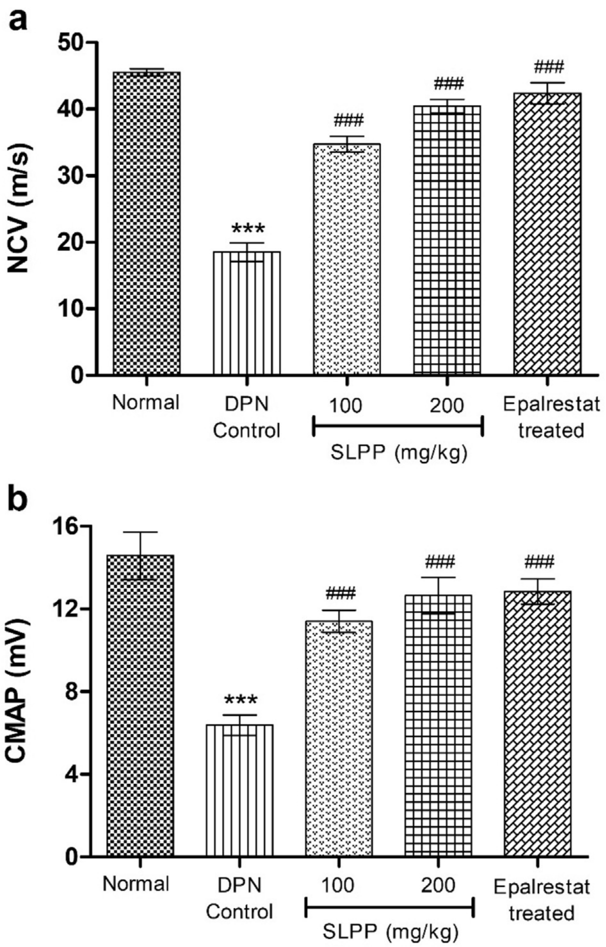

Fig. 1. Effect of *Symphyocladia latiuscula* polyphenols (SLPP) on the neuromuscular electrophysiology of experimental animals rendered diabetic by intraperitoneal injection of streptozotocin (45 mg/kg b. w) and maintained for six weeks for the induction of peripheral neuropathy. The nerve conduction velocity (NCV) of the left tibial sciatic nerve (a) and compound muscle action potential (CMAP) of the gastrocnemius muscle (b) were measured after treatment for 30 days. Epalrestat, an AR inhibitor, was used as a positive control. Values are expressed as mean ± SEM (n = 6). $^{***}p < 0.001$ compared with normal control, $^{\#\#\#}p < 0.001$ compared with DPN control.

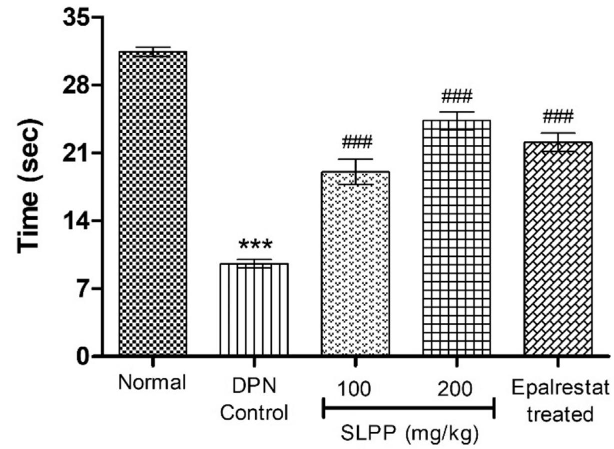

Fig. 2. Effect of SLPP on muscular grip strength of diabetes-induced neuropathic animals. The animals were placed on a rotating rod (20 rpm) and their residence time on it is considered as an index of muscle weakness/strength. Epalrestat was used as a positive control. Values are expressed as mean ± SEM (n = 6). $^{***}p < 0.001$ compared with normal control, $^{\#\#\#}p < 0.001$ compared with DPN control.

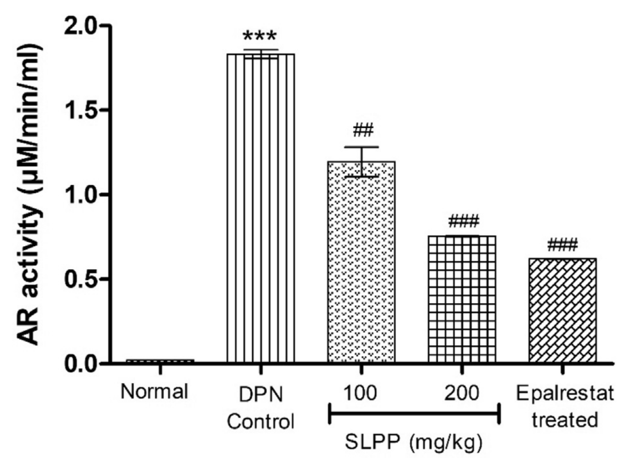

Fig. 3. Effect of SLPP on aldose reductase activity in the sciatic nerve of diabetic animals. Full length tibial sciatic nerves were isolated from the respective animal groups and homogenized. The AR enzymatic activity in the homogenates of respective groups was determined. Epalrestat, an AR inhibitor, was used as a positive control. Values are expressed as mean ± SEM (n = 6). $^{***}p < 0.001$ compared with normal control, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p < 0.01$ compared with DPN control.

3. Results

3.1. *Effect of Symphyocladia latiuscula polyphenols (SLPP) on NCV and CMAP*

The concentration of polyphenols extracted from *Symphyocladia latiuscula* was found to be 1.963 mg/mL. Peripheral neuropathy is characterized by lowering of nerve conduction velocity. The results on sciatic NCV measured 6 weeks after STZ injection showed significant reduction (18.50 ± 1.402 m/s, $p < 0.001$) when compared to the normal control (45.52 ± 0.555 m/s). When SLPP was administered according to the therapeutic dose (100 & 200 mg/kg) and schedule (for 4 weeks post induction of peripheral neuropathy), the NCV in diabetic group improved to 34.73 ± 1.213 and 40.44 ± 1.103 m/s respectively while that of epalrestat group to 42.41 ± 1.582 m/s (Fig. 1a). We also evaluated the CMAP of the gastrocnemius muscle. As shown in Fig. 1b, a significant ($p < 0.001$) restoration was observed in the groups treated with SLPP (100 & 200 mg/kg) and epalrestat as compared to the DPN control, the improvement in SLPP-treated group (11.39 ± 0.540 and 12.65 ± 0.882 mV) being almost identical to that of the standard drug, epalrestat-treated group (12.84 ± 0.608 mV).

3.2. *Effect of SLPP on muscular grip strength*

The measure of muscular grip strength is expressed as the duration, in seconds, of residence of the animal on the rotating rod till its fall. The time of residence was significantly reduced in the DPN control group (9.567 ± 0.433 s, $p < 0.05$) against the normal control (31.42 ± 0.475 s) after 6 weeks of STZ injection (Fig. 2). After 4 weeks of treatment with SLPP (100 & 200 mg/kg), the animals demonstrated a significant improvement (19.02 ± 1.311 and 24.30 ± 0.922 s, $p < 0.05$) in residence time on the rotating rod. A similar response was observed within the group administered with the standard drug epalrestat (22.08 ± 0.936 s, $p < 0.05$).

3.3. *Effect of SLPP on aldose reductase activity*

To assess the effect of SLPP on aldose reductase, the first rate-limiting enzyme in the polyol pathway of glucose metabolism, an assay of sciatic

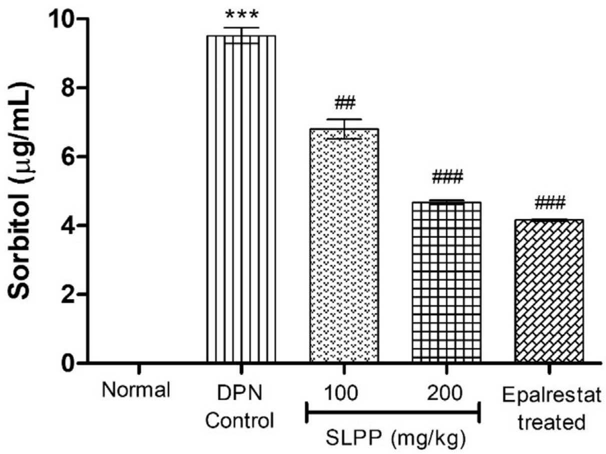

Fig. 4. Effect of SLPP on intraneural accumulation of sorbitol in the sciatic nerves of diabetic animals. Sorbitol concentration in the nerve homogenates was estimated by analytical high performance liquid chromatography (HPLC) employing a gradient program of mobile phase (CH_3CN and H_2O) with a flow of 1.0 mL min^{-1} against a standard sorbitol solution (100 µg mL^{-1}). Sorbitol concentration was determined by quantifying the AUC of sorbitol peak. Values are expressed as mean ± SEM (n = 6). ***$p < 0.001$ compared with normal control, $^{###}p < 0.001$, $^{##}p < 0.01$ compared with DPN control.

nerve AR activity was performed in all the experimental groups. As expected, Fig. 3 shows about 83-fold increment in the sciatic nerve aldose reductase activity in the DPN control group (1.832 ± 0.0254 µM/min/ml) when compared to the normal animals (0.0221 ± 0.00018 µM/min/ml). However, this elevated AR activity was attenuated by 1.5 fold (1.195 ± 0.0885 µM/min/ml; $p < 0.05$) and 2.4-fold (0.7535 ± 0.0026 µM/min/ml; $p < 0.001$) after treatment with SLPP (100 & 200 mg/kg) when compared to the DPN control. Even treatment with epalrestat exhibited a 3-fold reduction in AR activity (0.6202 ± 0.0015 µM/min/ml). Thus, the findings clearly establish significant AR inhibitory potential of SLPP.

3.4. *Effect of SLPP on intraneural accumulation of sorbitol*

DPN control rats displayed significant ($p < 0.05$) accumulation of intraneural sorbitol (9.515 ± 0.227 µg/mL) compared with nondiabetic mice (Fig. 4). After treatment with SLPP (100 mg/kg) a moderate reduction (6.807 ± 0.283 µg/mL) was witnessed ($p < 0.05$) whereas with 200 mg/kg dose a considerable (4.674 ± 0.065 µg/mL) reduction was observed ($p < 0.001$) which was comparable to the effect produced by epalrestat treatment (4.127 ± 0.019 µg/mL). Hence, the results suggest prevention of intraneural accumulation of sorbitol with SLPP treatment, a fact, that stems from the previous finding that established SLPP as an AR inhibitor.

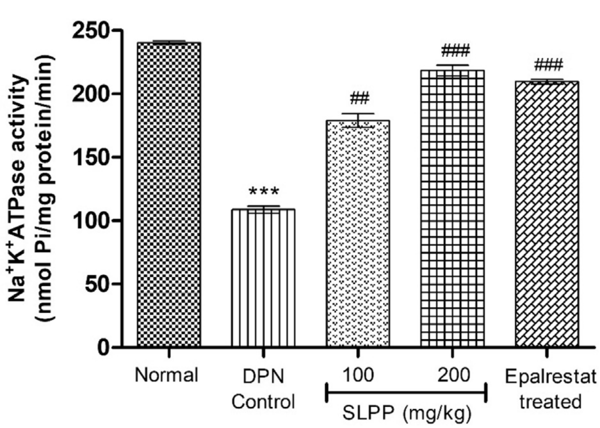

Fig. 5. Effect of SLPP on the Na^+ K^+-ATPase activity in the sciatic nerves of diabetic animals. The enzymatic activity in the nerve homogenate was assayed spectrophotometrically by determining the inorganic phosphate (Pi) released from ATP, in the presence and absence of ouabain, a specific Na^+ K^+-ATPase antagonist. Values are expressed as mean ± SEM (n = 6). ***$p < 0.001$ compared with normal control, $^{###}p < 0.001$, $^{##}p < 0.01$ compared with DPN control.

3.5. *Na^+ K^+-ATPase activity*

Na^+ K^+-ATPase activity was decreased by 2.2-folds in sciatic nerves of the DPN control animals (108.8 ± 2.813 nmol Pi/mg protein/min) compared to those of the normal control animals (240.4 ± 1.382 nmol Pi/mg protein/min). After treatment with SLPP (100 and 200 mg/kg) the Na^+ K^+-ATPase activity in the DPN control animals was increased by 1.6- and 2-folds (178.8 ± 5.355 and 218.4 ± 4.227 nmol Pi/mg protein/min) respectively. The extent of restoration in the enzyme activity with SLPP high-dose (200 mg/kg) was observed to be more than the epalrestat treated group (209.6 ± 1.732 nmol Pi/mg protein/min) (Fig. 5).

3.6. *Cytokine ELISA*

To understand the effects of SLPP on high glucose-induced production of proinflammatory cytokines (IL-6, IL-1β and TNF-α), ELISA was performed on the sciatic nerve lysate. As the SLPP high-dose (200 mg/kg) had consistently produced maximum effect, the remaining experiments were conducted with high-dose, henceforth. The production of all three pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) was increased by 7- to 8-folds in the nerves of DPN control animals compared to those from normal control animals (Fig. 6) while a significant reduction ($p < 0.05$) was observed post SLPP treatment. Reduction in the cytokine production after epalrestat treatment, however, was less significant ($p < 0.5$) compared to the DPN control.

3.7. *Western blot analysis*

As shown by Western blot analysis, expressions of AR and PKC were significantly increased in the DPN control animals when compared to the normal animals (Fig. 7). However, this expression was markedly attenuated in animals treated with SLPP and epalrestat. Convincingly, the effects of SLPP were found to be comparable to that of the specific AR inhibitor, epalrestat.

4. Discussion

Diabetic peripheral neuropathy represents a state of complication

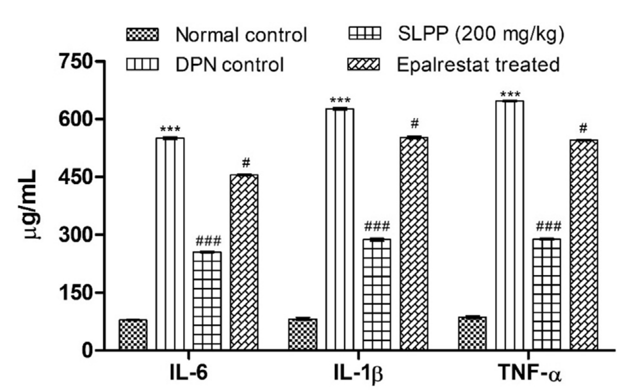

Fig. 6. Effect of SLPP on the expression of the pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) in the sciatic nerves of diabetic animals. Production of cytokines in the diabetic animals before and after treatment with SLPP were measured by ELISA. Values are expressed as mean ± SEM (n = 6). ***$p < 0.001$ compared with normal control, $^{###}p < 0.001$, $^{\#}p < 0.05$ compared with DPN control.

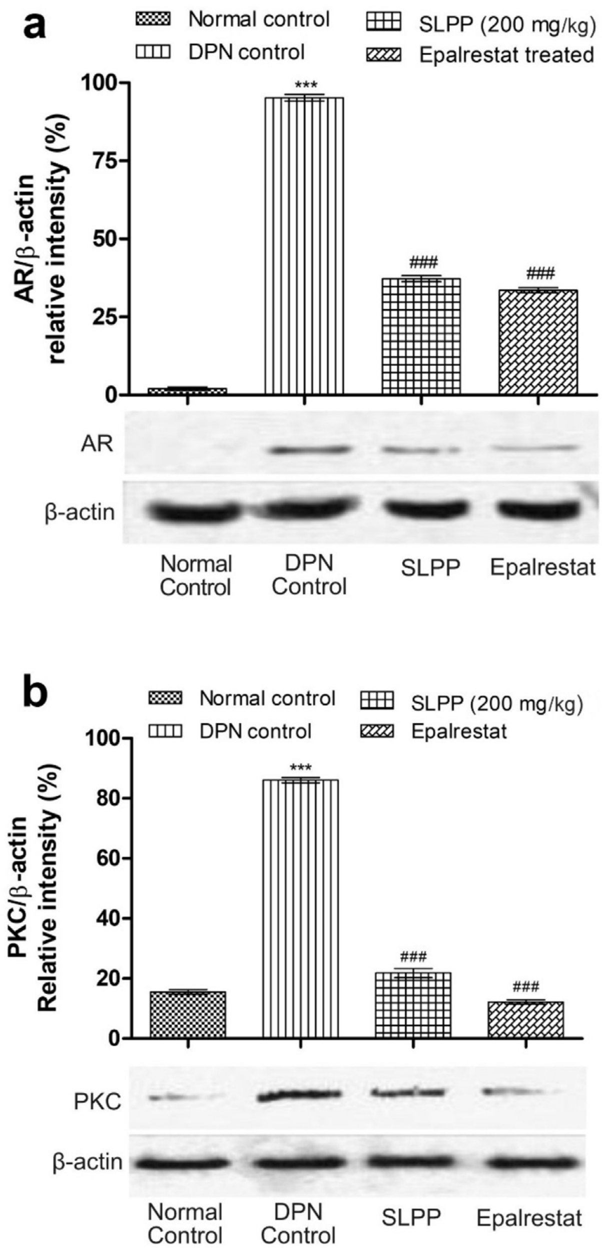

Fig. 7. Effect of SLPP on the expression of aldose reductase (a) and protein kinase C (b) in the sciatic nerves of diabetic animals. The levels of expression of AR and PKC were detected by western blot analysis and normalized to β-actin. Values are expressed as mean ± SEM (n = 6). ***$p < 0.001$ compared with normal control, ###$p < 0.001$ compared with DPN control. *The full blot images are not available.*

associated with chronic hyperglycaemia affecting all peripheral nerves including sensory and motor neurons characterized by pain, paresthesia, hyperesthesia, dysesthesia, proprioceptive defect, loss of sensation, muscle weakness and atrophy [40]. The polyol pathway has been identified as a major contributor in the development of neuropathy. Reduced conduction velocity has been found to develop in motor and sensory nerves in diabetic animals [41]. The conduction deficit has been prevented or reversed by treatment with an aldose reductase inhibitor [42].

Marine algae are one of the richest sources of structurally diverse natural products. In recent years, an increasing number of novel compounds have been isolated from marine algae and many of them have been reported to possess different biological activities [43, 44, 45, 46]. The red alga *Symphyocladia latiuscula* (Rhodomelaceae) is known to contain high concentrations of bromophenols that were reported to possess α-glucosidase inhibition activity [26], thereby owning the ability to control post-prandial hyperglycaemia, one of the approaches considered for treating diabetic patients [47, 48]. The bromophenols inhibit α-glucosidase activity with mixed or competitive inhibition mode. The interaction induces minor conformational changes of the enzyme, the hydrophobic and hydrogen bonds being the major driving forces of the interactions [49]. Bromophenols of Rhodomelaceae family were also reported to possess aldose reductase inhibition activity which may be beneficial in ameliorating neuropathic pain in peripheral neuropathy [45].

In the present study, the protective role of SLPP in experimental diabetic neuropathy was explored. To accomplish this, firstly, we studied the ability of SLPP to improve the neuromuscular electrophysiology (NCV of the sciatic nerve and CMAP of the gastrocnemius muscle) and muscular grip strength of STZ-induced diabetic animals. The results show significant restoration of NCV and CMAP, and muscle grip strength in animals treated with SLPP indicating its ameliorating effect. In experimental diabetic neuropathy, dramatic decrease in NCV has been widely reported [50, 51]. The deficit in Na^+K^+-ATPase activity has serious implications in nerve physiology. Decreased sciatic nerve Na^+K^+-ATPase activity has been found to alter the normal membrane axon repolarization after the repolarization induced by an action potential resulting in decreased NCV [52, 53]. Treatment with SLPP, in the present study, has significantly restored the sciatic nerve Na^+K^+-ATPase activity. Similar results were obtained reduced Na^+K^+-ATPase activity was normalized in STZ-induced diabetic rats treated with pre-germinated brown rice [54].

Aldose reductase overactivity has been implicated in hyperglycaemia that leads to the accumulation of sorbitol in the peripheral nerves that eventually causes neuropathic pain, a characteristic in diabetic neuropathy [8, 9]. This overactivity of AR was also been found to be in correlation with the decreased Na^+K^+-ATPase activity and motor nerve conduction velocity (MNCV) of the caudal nerves in STZ-diabetic rats. Strategic treatment with an AR inhibitor elevated Na^+K^+-ATPase activity and normalized the reduced MNCV [55]. Our study revealed significant reduction in the sciatic nerve AR activity after the treatment of neuropathic rats with SLPP. Sorbitol accumulation, a consequence of AR over-activity, was also lowered considerably in the sciatic nerves of animals treated with SLPP. The attenuation of these two polyol pathway over-activity of SLPP was found to be comparable to that of epalrestat which suggests that SLPP is a potential AR inhibitor.

The overexpression of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) in diabetic neuropathy directly increases nerve excitability and damage myelin leading to oedema and further infiltration by immune cells [56]. Production of IL-1β in injured nerves has directly been found to sensitize nociceptors in primary afferent neurons [57]. IL-6 induces pain directly by increasing the sensitivity of nerve endings [58] and can also enhance neuropathic pain in the dorsal horn through the activation of STAT3 signalling pathway as it is the key mediator of signal transduction of pain in glial cells after peripheral injury [59]. Evidence exists that inhibition of cytokine production has proven beneficial in the management of neuropathic pain and neuroinflammation [60]. Our results demonstrated significant reduction in the production of all the three pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) in the sciatic nerve of neuropathic rats as compared to the diseased animals in a dose-dependant manner.

Hyperglycaemia serves as a key signalling event in the activation of the protein kinase C (PKC) family of protein kinases [61]. The contribution of PKC to diabetic neuropathy is through neurovascular mechanisms such as blood flow and conduction velocity. There are immunochemical evidences for the presence of PKC-α, -β1, -β2, -γ, -δ and -ε isoforms in nerve [62, 63]. It was found that overexpression of PKC was involved in the reduction of Na^+-K^+-ATPase activity, leading to decreased nerve conduction and regeneration. The manifestations were normalised upon treatment with a non-selective PKC inhibitor [64, 65]. The contribution of polyol pathway to high glucose-induced PKC activation has been studied by investigators. Aldose reductase overactivity has been implicated in hyperglycaemic activation of PKC [66]. Inhibition of AR by tolrestat (AR inhibitor) prevented high glucose-induced activation of PKC in cultured vascular smooth muscle cells (VSMCs) isolated from rat aorta. Also, ablation of aldose reductase gene using RNA interference, to exclude the non-specific effects of AR inhibitors [67], reduced AR protein to undetectable levels and consequently, prevented high glucose-induced activation of PKC. High glucose has been found to stimulate the membrane translocation of conventional (α, β1, β2 and γ) and novel (δ and ε) isoforms of PKC, the most significant being the PKC-β (β1 and β2) and -δ isoforms followed by enhancement in their phosphorylation. Treatment with AR inhibitors prevented both high glucose-induced membrane translocation and phosphorylation of the PKC isoforms. The AR inhibitors also prevented the increase in hyperglycaemia induced-diacylglycerol (DAG) synthesis from phospholipids and also abrogated phospholipase C (PLC) phosphorylation, an event essential for DAG synthesis [68]. However, aldose reductase inhibition did not inhibit phorbol-12-myristate-13-acetate (PMA)-induced membrane translocation of PKC, suggesting that inhibition of aldose reductase does not prevent PKC activation directly, but prevents DAG synthesis through inhibition of PLC phosphorylation [69, 70]. Our Western blotting results, in absolute harmony with the preceding discussion, reveal significant reduction in the expression of PKC and AR proteins in the sciatic nerves of diabetic animals treated with SLPP as compared to the diabetic animals, thus clearly indicating its decisive role of aldose reductase inhibition.

5. Conclusion

The neuroprotective role of the polyphenols of the red alga *Symphyocladia latiuscula* was evaluated in experimental diabetic peripheral neuropathy. We found that SLPP improved the electrophysiological parameters (NCV and CMAP) and muscular grip strength of animals with diabetic neuropathy. It also reduced aldose reductase activity and its expression, and consequently, prevented the accumulation of sorbitol in the sciatic nerves of diabetic animals. The $Na^{+}K^{+}$-ATPase activity was restored significantly and the production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) was reduced as well. Finally, the expressions of aldose reductase and PKC proteins were also attenuated after treatment of neuropathic animals with SLPP. These findings suggest that the polyphenols of *Symphyocladia latiuscula* may find use in the treatment of diabetic peripheral neuropathy. However, clinical trials must be performed to assess the therapeutic efficacy in human beings.

Declarations

Author contribution statement

Suman Samaddar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Raju Koneri: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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